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Myogenic Determination Factor Expression in the Developing Avian Limb Bud: An RT–PCR Analysis

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This study examines the time of appearance of myogenic determination factor (MDF) transcripts in developing chick limbs and other embryonic tissues using reverse transcription–polymerase chain reaction (RT–PCR). In this assay, PCR amplification profiles of cDNA samples from stage 15–26 limb buds are compared to those of E11 heart, a tissue operationally defined as containing background levels of MDF transcripts which are thought to be nonfunctional. Myf 5 and MRF 4 transcripts are detected in stage 15 forelimbs, which is the earliest stage limb in which myogenic precursors have been detected following their migration from the somite. In contrast, MyoD and myogenin transcripts do not appear until about 1.5 days later, closer to the time of cytological muscle differentiation. A survey of MyoD, myf 5, and MRF 4 transcripts in other embryonic tissues reveals that MyoD transcripts are distributed in a highly skeletal muscle-specific pattern. In contrast, myf 5 and MRF 4 mRNAs are detected at significant levels in embryonic tissues that do not contain muscle or muscle precursor cells such as stage 9–15 neural tubes and stage 9–12 lateral plates, while these transcripts are found at very low levels in E11 heart and liver. The RT–PCR results are compared to those from *in situ* hybridization experiments as well as from cell culture assays of the myogenic potential of early limb cells.

INTRODUCTION

Myogenic determination factors (MDFs) have been shown to be important for skeletal muscle determination and differentiation. This study examines the developmental sequence of MDF expression in the chick limb bud and attempts to correlate this information with the overall problem of how the limb myogenic lineage becomes established. Transplantation and *in vitro* analyses of avian limb bud myogenesis have demonstrated that cells capable of muscle differentiation are present well before cytologically detectable limb muscle differentiation (see Stockdale, 1992; Hauschka, 1994, for reviews). Similarly, premyogenic cells can be detected in mouse limb explant cultures more than a day before MDF transcripts can be found within the developing limb buds by *in situ* hybridization (Sassoon, 1989). To achieve a more sensitive analysis of *in vivo* MDF gene expression, we have developed a reverse transcription–polymerase chain reaction (RT–PCR) assay to examine when MDF transcripts can be detected in the developing chick limb bud.

The limb and body wall muscles derive from the lateral half of the somite subsequent to dermomyotome formation (see Ordahl, 1993, for review). In the chick embryo, cells begin to leave the ventrolateral edge of the dermomyotome at stage 14 and then migrate into the forelimb bud mesoderm which is derived from the lateral plate (Christ *et al.*, 1977). Muscle precursor cells can be detected in the forelimb bud by stage 15 (Seed and Hauschka, 1984). In a similar manner, more caudal somitic cells at the hindlimb level begin to migrate at stage 15 and probably are present in the hindlimb bud at stage 16 (Jacob *et al.*, 1979).

Other cell culture assays and transplantation studies have examined when cells committed to the muscle lineage can be found in the limb bud and, in addition, have identified subpopulations of early limb myoblasts by their fusion properties, growth requirements, and myosin heavy chain isoform expression (Bonner and Hauschka, 1974; White *et al.*, 1975; Rutz and Hauschka, 1982; Miller and Stockdale, 1986; Seed and Hauschka, 1988; Van Swearingen and Lance-Jones, 1995). Most studies examining myogenic populations in the limb have entailed the growth and differentiation of muscle precursor cells in culture and have used relatively late markers of muscle differentiation to identify cells in the muscle lineage. More recently, it has been found by *in situ* hybridization that Pax 3 expression marks the migratory cells of the somite, but no MDF transcripts have been de-

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tected in these cells (Bober *et al.*, 1994; Williams and Ordahl, 1994).

MDFs (MyoD, myogenin, myf 5, and MRF 4) are transcription factors that are clearly involved in the process of muscle differentiation (see Edmondson and Olson, 1993, for review). Expression of the MDF genes is hypothesized to play an early mechanistic role in skeletal muscle development, since *in situ* hybridization studies of mouse and chick embryos indicate that MDF expression occurs at times and in locations in which the skeletal muscle lineage is being established. A role for MDFs in determining the myoblast lineage is also supported by results from mice containing double null mutations of myf 5 and MyoD, in which there was a complete absence of muscle (Rudnicki *et al.*, 1993). Results from chimaeric mouse embryos containing cells with the lacZ gene in the myf 5 locus have shown no β-galactosidase staining in cells in the area between the somite and the limb bud, supporting the hypothesis that the premyogenic cells do not express MDFs until they reach the permissive environment of the limb bud (Tajbakhsh and Buckingham, 1994).

MDFs are expressed sequentially during development, suggesting different roles for each factor during muscle development in the embryo. *In situ* hybridization studies by Pownall and Emerson (1992) examined the expression of qmf 1, 2, and 3 (quail homologues of MyoD, myogenin, and myf 5) in stages 12 and 13 quail embryos. By analyzing expression with respect to the rostral–caudal position of the somites, they determined that MyoD is expressed first; MyoD transcripts were detected in the younger, more caudal somites, while myf 5 transcripts colocalized with MyoD transcripts only in the older, more rostral somites of stage 12. Myogenin transcripts were not detected in somites until stage 13. In avian limb buds, MyoD transcripts were first detected at stage 24 (Charles de la Brousse and Emerson, Jr., 1990; Williams and Ordahl, 1994). The sequence of MDF gene expression in the avian limb bud has not been examined extensively by *in situ* hybridization, so it is unknown whether the different factors are expressed in a sequence similar to that in the somite. *In situ* hybridization and RT–PCR analyses of developing mouse somites and limb buds have revealed a different sequence of MDF expression from chick embryos. Myf 5 transcripts were detected by *in situ* hybridization in the earliest mouse somites followed by myogenin, MRF 4, and finally, MyoD. In the mouse limb bud, myf 5 was again the first MDF transcript detected, and MyoD and myogenin were coexpressed in later stages (Sassoon *et al.*, 1989; Ott *et al.*, 1991). MRF 4 mRNA was detected 2 days later than MyoD and myogenin mRNA in the mouse limb (Bober *et al.*, 1991; Hinterberger *et al.*, 1991). However, RT–PCR with mouse MRF 4 primers detected transcripts in limb stages containing myf 5 but not MyoD and myogenin transcripts (Hannon *et al.*, 1992). The RT–PCR data with the other mouse MDF primers in the somite and the limb were similar to *in situ* hybridization data, although the times of activation were detected slightly earlier with RT–PCR.

In this study, we have used an RT–PCR analysis to investigate MDF expression in the developing chick limb bud and other embryonic tissues. We chose to use RT–PCR because its extreme sensitivity permits detection of very low mRNA levels in small numbers of cells. The population of migrating myogenic precursors consists of only several thousand cells (Charles Ordahl and Bodo Christ, personal communication), and initial expression of MDF genes in these cells is probably low. The MDF expression data were then compared with earlier data from culture experiments that have indicated when different populations of premyogenic cells are found in the limb. These comparisons provide insights as to possible relationships between cell determination state, myoblast diversity, and the expression of MDF genes.

MATERIALS AND METHODS

Eggs. Chick embryos were obtained from fertilized White Leghorn eggs (H and N International, Redmond, WA) which had been incubated in a forced draft incubator at 37–38°C, 100% humidity. Developmental stages were ascertained according to the criteria of Hamburger and Hamilton (1951).

Embryo dissection and RNA extraction. Embryos were dissected in cold, diethyl pyrocarbonate (DEPC)-treated, phosphate-buffered saline. Forelimbs, hindlimbs, hearts, livers, and other embryonic tissues were removed from single or multiple embryos using electrolytically sharpened tungsten needles (Fig. 1). Distal and proximal fore- and hindlimb samples were obtained from stage 25/26 embryos (Fig. 1D). The distalmost quarter of the fore- or hindlimb was dissected away from the remaining proximal portion of the limb using tungsten needles. Lateral plate tissue and neural tubes were obtained from embryos after trypsin treatment of the embryos (Fig. 1A). These embryos were removed from extraembryonic membranes in 4°C Ham's F10C (F10 supplemented with 0.8 m*M* Ca^{2+} and 0.05 mg/ml gentamicin, Sigma) and were pinned with tungsten wires to a dissection dish containing Sylgard 184 elastomer (Dow Corning) blackened with activated charcoal. After removal of the F10C, 2 mg/ml trypsin (Worthington) at room temperature was applied to cover the embryos (100–200 μl). The ectoderm of the embryo was removed with tungsten needles, and the lateral plate and neural tube were then separated from the somites. For all samples, the tissue was dissolved in 500–1000 μl of 4 *M* guanidine thiocyanate/8% β-mercaptoethanol solution. Total RNA was isolated essentially as described in Chomczynski and Sacchi (1987), except that the first precipitation was performed at −20°C with an equal volume of isopropanol for a minimum of 16 hr, followed by a 30-min 45,000-rpm spin at 4°C in a Beckman tabletop ultracentrifuge (TL100). The RNA pellets were then processed as in Chomczynski and Sacchi (1987), and each RNA sample was quantified spectrophotometrically at 260 nm.

RT–PCR. One microgram of total RNA was used for RT–PCR for all primer pairs except for the cytoplasmic β-actin primers, for which 100 ng of total RNA was used. The RNA was denatured briefly with 1 unit of inhibit-ACE (5prime3prime, Inc.) and 0.2 μg oligo $(dT)_{(15)}$ primer (Promega) at 65°C for 5 min. Then 1× PCR buffer (Promega), 1.5 m*M* $MgCl_2$, 1 m*M* each dNTP, and 200 units MMLV Reverse Transcriptase (Gibco-BRL Life Technologies, Inc.) were added to a final reaction volume of 20 μl. Reverse transcrip-

TABLE 1
PCR Primers

Primer	Primer sequence	cDNA regions
MyoD (CMD1)[a]		
Pair ''a''	Forward: TACCCAGTGCTGGAGCACTA	Identical to nt 641–660
	Reverse: GTCTTGGAGCTTGGCTGAAC	Complementary to nt 1092–1111 in 3′ untranslated region
Pair ''b''	Forward: GGACATGCACTTCTTCGAGG	Identical to nt 250–269
	Reverse: TCTCCTGCGAGAGCTACAGG	Complementary to nt 732–751
Myogenin[b]	Forward: ACGAGCCTCAACCAGCAGGA	Identical to nt 432–451
	Reverse: TCTGCCTGGTCATCGCTCAG	Complementary to nt 591–610
Myf 5[c]		
Pair ''a''	Forward: AGGGAACAGGTGGAGAACTA	Identical to nt 552–573 in exon 1
	Reverse: TCATAGCGCCTGGTAGGTCC	Complementary to nt 1302–1321 in exon 3
Pair ''b''	Forward: CCCTGAGGAAGAGGAACACG	Identical to nt 281–300 in exon 1
	Reverse: GATGCTGGAGAGGCAGTCCA	Complementary to nt 1164–1183 in exon 3
MRF 4[d]	Forward: AGGCTGGATCAGCAGGACAA	Identical to nt 459–478
	Reverse: CACATTTCTCCACCGCCTCT	Complementary to nt 782–741
Cytoplasmic β-actin[e]	Forward: AATGAGAGGTTCAGGTGCCC	Identical to nt 3140–3159 in exon 4
	Reverse: ATCACAGGGGTGTGGGTGTT	Complementary to nt 4190–4209 in 3′ untranslated region

[a] Lin *et al.* (1989)
[b] Fujisawa-Sehara *et al.* (1990)
[c] Saitoh *et al.* (1993)
[d] Fujisawa-Sehara *et al.* (1992)
[e] Kost *et al.* (1983)

tion reactions were incubated for 10 min at room temperature, 60 min at 42°C, and 5 min at 95°C. The whole reverse transcription reaction was diluted to 100 μl final volume with 1× PCR buffer, 1–1.5 m*M* $MgCl_2$ (concentration dependent on primer pair used), 20 pmol each of forward and reverse primer, and 2 units of *Taq* polymerase (Promega). The MRF 4 primers and the ''a'' set of myf 5 primers were used in PCR reactions that contained a 1.0 m*M* $MgCl_2$ final concentration. All the remaining sets of primers used a 1.5 m*M* $MgCl_2$ concentration. Samples were overlaid with paraffin oil (~80 μl) and amplified in a Thermocycler (Eppendorf). The cycling parameters were as follows: the initial cycle consisted of a 95°C denaturation for 5 min, 1 min at a 55°C annealing temperature, and 1 min at a 74°C extension temperature. The remaining cycles were for 1 min each at 95°, 55°, and 74°C, with the final cycle having a 10-min extension at 74°C. The total number of cycles varied from 15 to 40 depending on the primer pair used. Fifteen-microliter samples of each reaction were removed every 2–5 cycles in the last 10–15 cycles. PCR products were analyzed by electrophoresis of 12.5 μl of each sample on a 3% NuSieve (FMC Corp.)/1% agarose gel, followed by ethidium bromide staining. Further verification of PCR product identity was determined by size, sequencing of PCR clones, Southern blot hybridization, or diagnostic restriction digests. The restriction enzymes used to verify PCR product identity yielded characteristic restriction fragments. *Pvu*II was used to digest PCR products from reactions containing myf 5, MyoD, or myogenin primers, and *Nco*I was used to digest MRF 4 PCR products.

All PCR primers were synthesized by the University of Washington Howard Hughes Chemical Synthesis Facility. Primers for genes whose genomic structure is known were designed such that the amplified sequence spanned introns. As the exon–intron structures of chick MyoD, myogenin, and MRF 4 have not been published, primers were designed to areas of the cDNA that would span introns in the homologous mouse sequence. Sequences of the primers are shown in Table 1.

Determination of PCR primer pair sensitivity. DNA fragments containing sequences of the MDFs were isolated from cDNA plasmids and used in PCR assays to quantify the range of target molecules that each primer pair can amplify in a given number of cycles. For the MyoD primers, the DNA used in the PCR was the 1.5-kb *Eco*RI insert of CMD1 in Bluescript KS (Lin *et al.*, 1989). An 800-bp EcoRI insert from a chick myogenin clone (Saitoh *et al.*, 1993) was used to test the myogenin primers. The target DNAs for myf 5 and MRF 4 were derived from *Eco*RI/*Bam*HI digests of pBS plasmids containing RT-PCR products cloned into the *Sma*I site of the plasmid. Each target DNA was quantified on a fluorimeter with Hoescht stain and diluted in water to give the appropriate number of copies of DNA. These dilutions were used in 100-μl PCR reactions under conditions identical to those described above for the RT–PCR, and the results of the PCR assay were analyzed identically to the RT–PCR studies. The amount of DNA used in each PCR reaction (i.e., the number of target molecules) was compared to the cycle number at which the product was first visible. This indicated the approximate number of target molecules that could

TABLE 2
Sensitivity of PCR Primers

Myogenic determination factor PCR primer	No. of PCR cycles	Range of the number of target molecules that can be detected[a]
Myf 5[d]	25	>1100[b], <1380[c]
	40	1–15
Myogenin	25	>5500, <11,000
MyoD[d]	25	>11,000, <30,000
MRF 4	25	>11,000, <30,000

[a] This range gives an approximation of how many target molecules are required to give visible product on an ethidium bromide stained agarose gel given a particular number of PCR cycles.

[b] The smaller number in the range represents the greatest number of target molecules tested that failed to amplify visible product.

[c] The greater number in the range represents the smallest number of target molecules tested that produced visible product following amplification.

[d] The ''a'' set of myf 5 and MyoD primers were used in these studies.

be detected at a given PCR cycle number for each of the different sets of primers.

RESULTS

Sensitivity of Myogenic Determination Factor PCR Primers

A pair of PCR primers for each of the four MDF cDNAs was used in PCR assays with known quantities of plasmid derived target DNA to determine the approximate numbers of target molecules the PCR primers were capable of amplifying (Table 2). For each primer pair the smaller number in the range represents the greatest number of target molecules tested that failed to produce visible product. The greater number in the range of target molecules represents the smallest target number tested that produced visible product following amplification.

The four sets of MDF PCR primers exhibited different sensitivities. The myf 5 primers appear to be the most sensitive since 25 PCR cycles were sufficient to amplify approximately 1400 target sequences. In fact, the myf 5 primers can amplify a single target sequence with 40 PCR cycles. The remaining MDF primers required greater than 5500 target molecules in order to produce visible product after 25 cycles. Myogenin PCR primers required 11,000 targets with 25 PCR cycles, while MyoD and MRF 4 primers required about twice this number of target sequences, indicating that the myogenin primers are more sensitive than the MyoD and MRF 4 primers. Although the myogenin, MyoD, and MRF 4 primers are less sensitive than the myf 5 primers, RT–PCR reactions with these primers did not require further optimization, since the sensitivity of the reactions was sufficient to detect background levels of these transcripts in nonskeletal muscle tissues (see subsequent sections).

Myogenic Determination Factor Transcript Detection in Developing Embryonic Chick Limb Buds

RT–PCR analysis was used to detect MDF mRNAs in chick embryo fore- and hindlimbs (Figs. 1B and 1C) from single or multiple embryos of various stages using the primers in Table 1. A profile of the PCR amplification for each cDNA was obtained by removing a sample every 2–5 cycles during the last 10–15 PCR cycles. The total number of cycles was dependent on the primer pair used since different primer pairs detect background transcripts after different cycle numbers. In the analysis of each RT–PCR experiment, the minimum number of cycles that were required to visualize the correct PCR product on a gel from a nonskeletal muscle tissue (E11 heart) was determined. This minimum number was then operationally defined as the cycle number that detects background transcript levels from the MDF gene being examined, since MDF mRNAs are not detected in heart by *in situ* hybridization analyses (Buckingham *et al.*, 1992) or by Northern analysis (e.g., MyoD (Davis *et al.*, 1987)). E11 liver also exhibits similar background levels of MDF transcripts (see below, Table 4, and Figs. 4 and 5). If the limb sample run in parallel with the E11 heart sample showed a similar PCR amplification profile to heart, or required a greater number of cycles before the specific PCR product was detected, then that sample was interpreted as containing background levels of the target mRNA, as operationally defined by this RT–PCR assay system. However, if the PCR product was detectable at a lower cycle number than heart, then the experimental sample was considered to have significant levels of the target mRNA.

An example of one MDF analysis is shown in Fig. 2 in which the ''a'' set of myf 5 primers was used to amplify cDNA generated from stage 15–22 forelimb buds and E11 heart. In this particular experiment, no background myf 5 transcripts were detectable in E11 heart even after 40 PCR cycles (Fig. 2A), suggesting that few if any myf 5 transcripts were present in E11 heart. The 360-bp myf 5 PCR product was observed in the stage 15–22 forelimb samples after as few as 27–29 PCR cycles, indicating significant levels of myf 5 transcripts in all of these samples. The ''b'' set of myf 5 primers was used in other experiments to verify RT–PCR results from the ''a'' set; similar results were observed. The myf 5 PCR products amplified are of the predicted size and are cut to the appropriate 342-bp fragment in diagnostic *Pvu*II restriction digests. The identity of the myf 5 PCR product obtained with these primers has also been confirmed by sequencing (data not shown). Cytoplasmic β-actin primers were also used in parallel reactions in the same experiment to verify the integrity and quantification of the RNA for each sample (Fig. 2B). All the cDNA samples exhibited efficient amplification of the 409-bp cytoplasmic β-actin PCR product after 20 cycles.

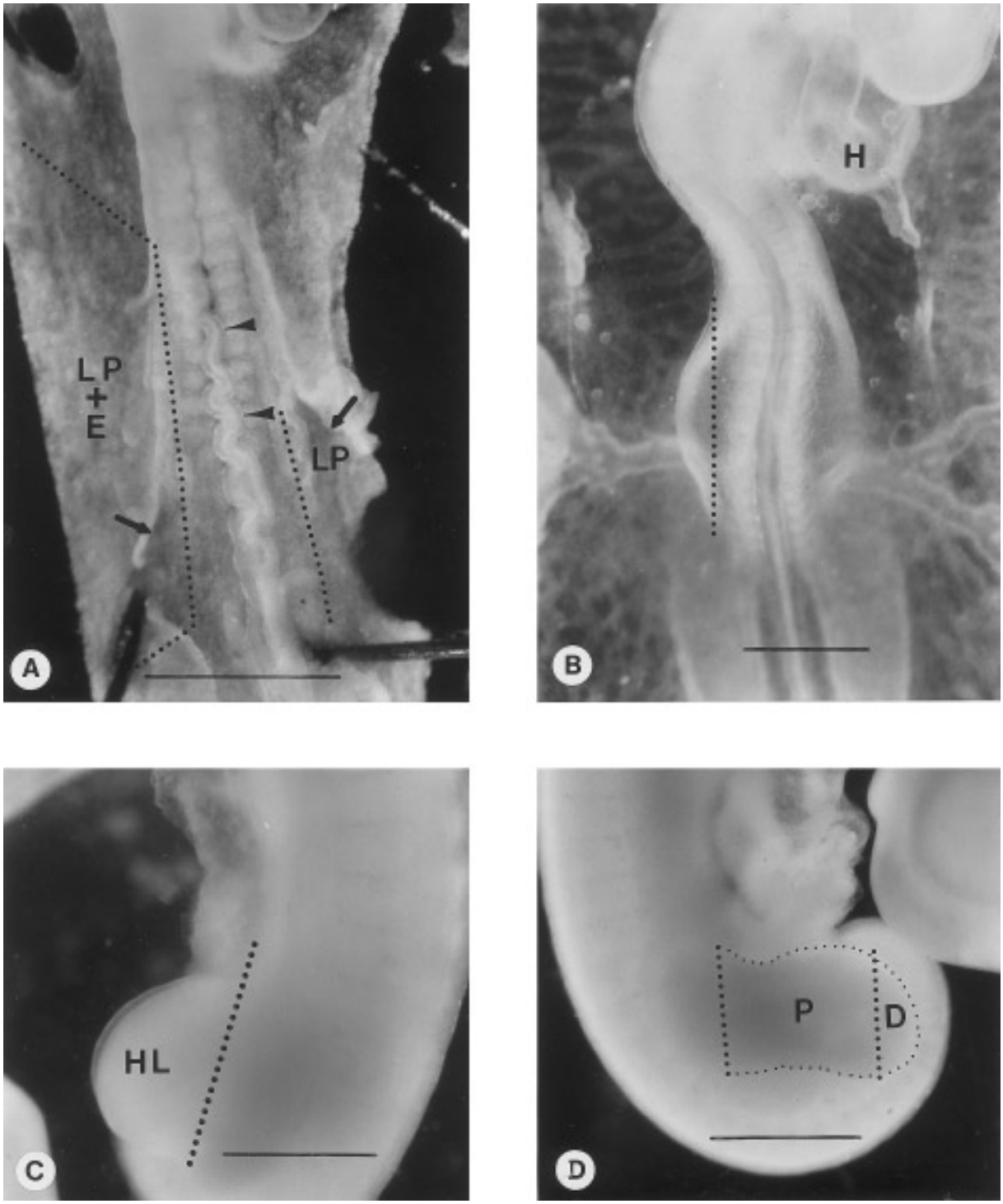

FIG. 1. Embryonic tissues dissected and used for RT–PCR assays. All embryos are oriented such that the rostral end of the embryo is at the top of the page. (A) Stage 12 embryo after treatment with trypsin and partial removal of the overlying ectoderm (E) is shown. Arrows (with points) indicate the edge of the ectoderm. In the dissection, lateral plate tissue (LP) was removed after removal of somites, segmental plate mesoderm, and neural tube (arrowheads). The dotted lines demarcate the approximate cuts made for the dissection for lateral plate tissue which includes not only mesoderm but the underlying endoderm. (B) Stage 15 embryo showing the area of forelimbs dissected (dotted line). H, heart. (C) Caudal half of a stage 23 embryo showing the approximate cuts made for the dissection of the hindlimb (HL). (D) Caudal half of a stage 25 embryo showing the approximate cuts (dotted lines) made during dissection of the distal quarter (D) and remaining proximal portion (P) of the hindlimb (outlined by dotted line). Bars, approximately 1 mm.

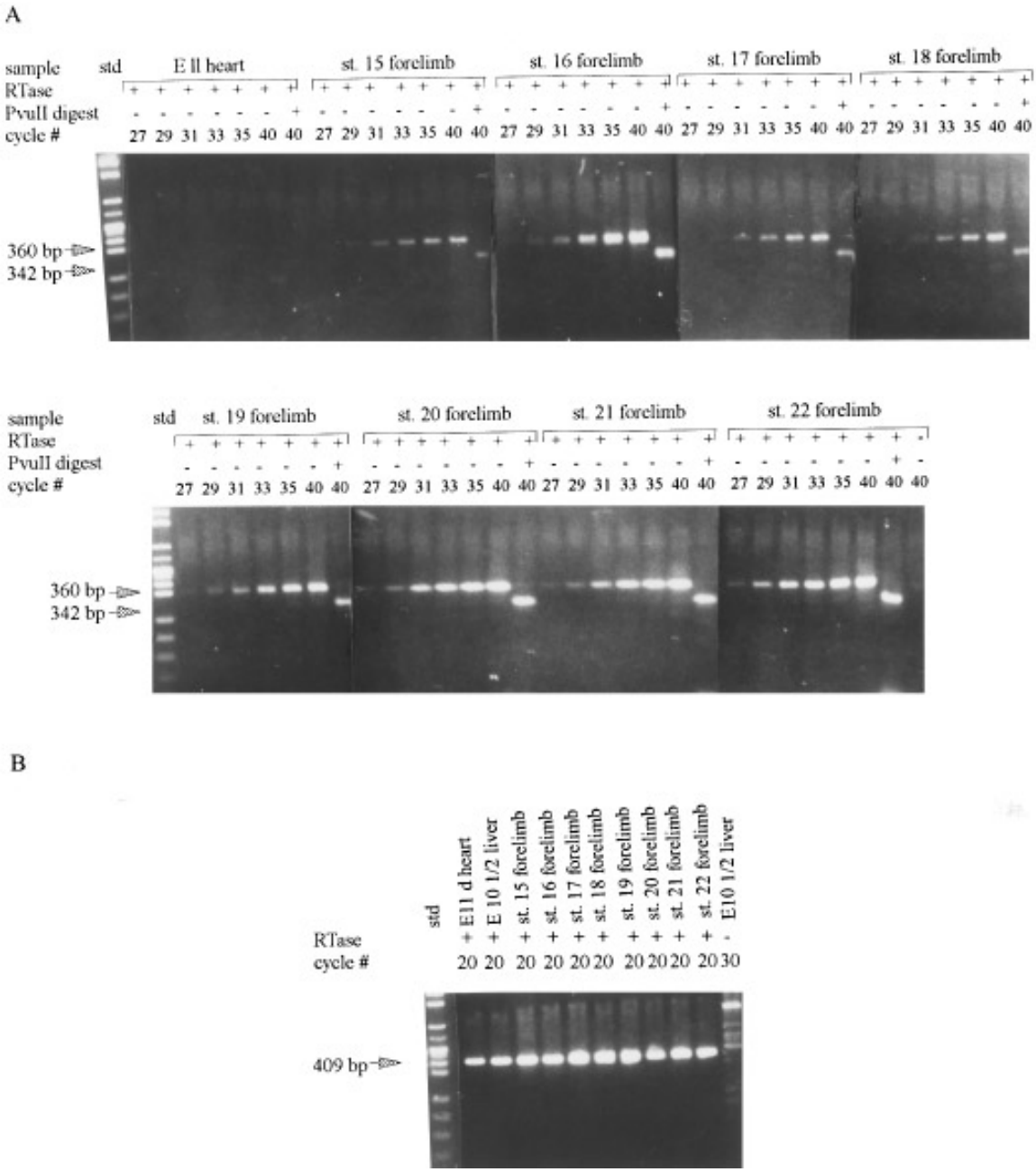

FIG. 2. Detection of myf 5 transcripts by RT–PCR in stage 15–22 chick forelimbs. The ''a'' set of myf 5 PCR primers (A) or cytoplasmic β-actin PCR primers (B) were used to amplify cDNA made from E11 heart and stage 15–22 forelimb total RNA. (A) Myf 5 transcripts were not detected in heart even after 40 PCR cycles. In stage 15–22 forelimb samples, the 360-bp PCR product was detectable by ethidium bromide staining with 40 or fewer cycles. Verification of the 360-bp PCR product as the targeted portion of cDNA was confirmed by a *Pvu*II digest of the 40-cycle samples to yield the predicted 342-bp product. The stage 22 forelimb control reaction that contained no reverse transcriptase (-RTase) failed to amplify product after 40 cycles. (B) The 409-bp cytoplasmic β-actin PCR product was detected from 100 ng of total RNA for all the samples after 20 cycles, thus verifying the integrity of RNA samples that exhibited no PCR product with myf 5 primers. Only nonspecific products were seen in the cytoplasmic β-actin minus reverse transcriptase control reaction after 30 cycles using liver (or any other sample).

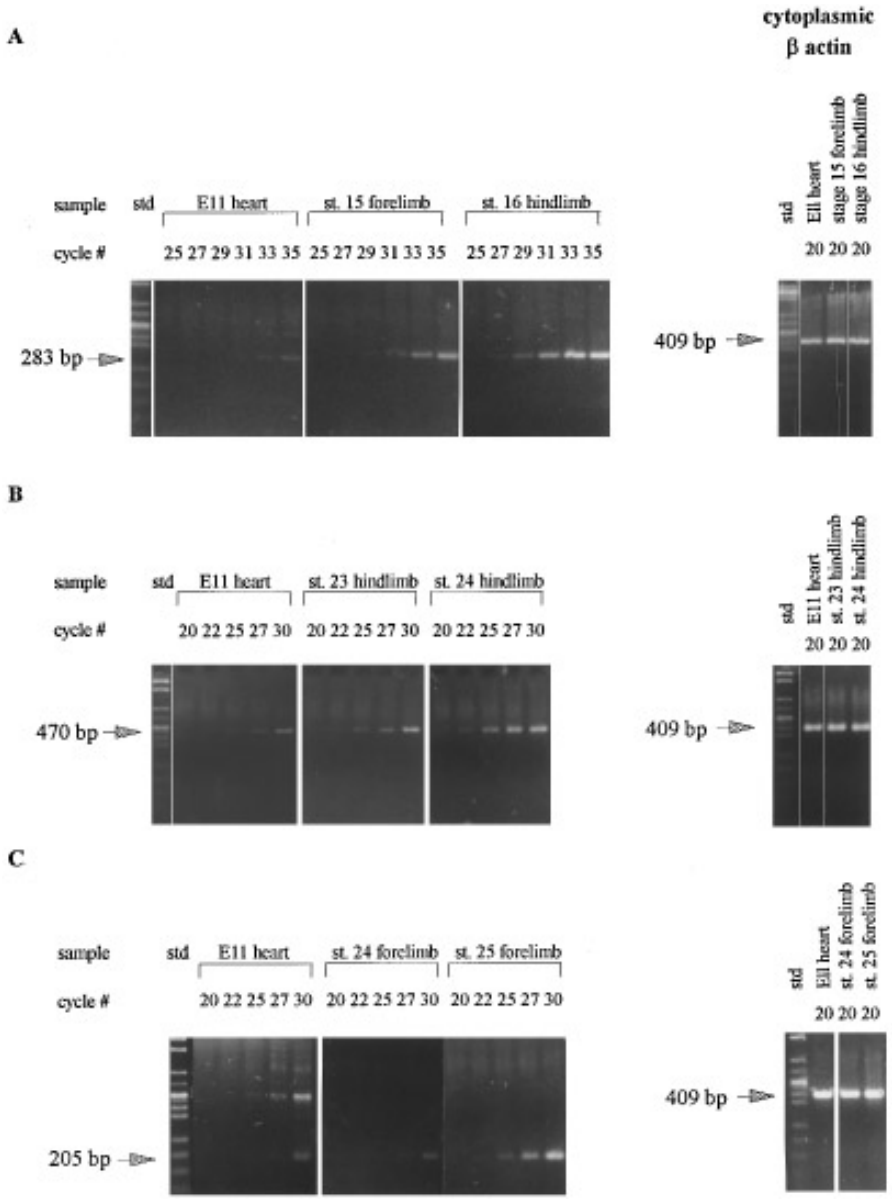

FIG. 3. Detection of MRF 4, MyoD, and myogenin transcripts by RT–PCR in various stages of fore- or hindlimbs. (A) MRF 4 and cytoplasmic β-actin PCR primers were used to amplify cDNA made from E11 heart, stage 15 forelimbs, and stage 16 hindlimbs. Background levels of the 283-bp product were detected in the heart sample after 33 cycles. The stage 15 forelimb and stage 16 hindlimb samples produced visible PCR product after 29 cycles. (B) The ''a'' set of MyoD and the cytoplasmic β-actin PCR primers were used to amplify cDNA made from E11 heart and stages 23 and 24 hindlimbs. The E11 heart and stage 23 hindlimb samples exhibited the 470-bp MyoD PCR product after 25 cycles, and 22 cycles of PCR were required to produce visible levels of MyoD product from stage 24 hindlimbs. Although MyoD PCR products appear in E11 heart and stage 23 hindlimb at the same cycle number, the intensity of the two bands may reflect a slightly higher MyoD mRNA level in stage 23 hindlimb. Some stage 23 hindlimb samples clearly contain MyoD mRNA levels above background, while others clearly contain background levels (Table 3). These differences between samples may reflect the transitional nature of MyoD activation. (C) Myogenin and cytoplasmic β-actin PCR primers were used to amplify cDNA from E11 heart and stages 24 and 25 forelimbs. The 205-bp myogenin PCR product was seen after 30 cycles in E11 heart and stage 24 forelimb samples, and the stage 25 forelimb sample exhibited myogenin PCR product after 25 cycles. The E11 heart reaction also contained a PCR product of higher molecular weight which is most likely due to nonspecific amplification with the myogenin primers. The identities of the MDF PCR products were verified by diagnostic restriction digests, and no specific products were detected in reactions without reverse transcriptase (data not shown). All cDNA samples exhibited efficient amplification of the 409-bp cytoplasmic β-actin product.

TABLE 3
Myogenic Determination Factor Transcript Detection in Limb Buds

Myogenic determination factor	Embryo stage	Forelimb		Hindlimb	
		No. of embryos examined[a]	No. of embryos showing expression[b]	No. of embryos examined[a]	No. of embryos showing expression[b]
Myf 5	→ 15[c]	5	5	[d]	[d]
	→ 16[e]	4	4	6	6
	17	4	3	4	4
	18	5	4	5	4
	19	3	3	3	3
	20	2	2	2	2
	21	2	2	2	2
	22	2	2	2	2
MRF 4	→ 15[c]	3	3	[d]	[d]
	→ 16[e]	4	3	4	4
	17	4	3	4	3
	18	3	3	3	3
	19	3	2	4	3
	20	5	2	6	4
	21	5	4	5	3
	22	5	4	4	3
	23	4	4	3	3
	24	3	3	4	3
	25	4	4	3	3
	26	3	3	3	3
MyoD	22	3	0	3	0
	→ 23[f]	4	2	4	2
	→ 24[f]	3	2	3	3
	25	3	3	3	3
Myogenin	21	2	0	N.D.[g]	N.D.
	22	2	0	3	0
	→ 23[e]	4	1	7	4
	24	6	2	6	4
	→ 25[c]	4	4	4	4
	26	2	2	2	2

[a] Greater numbers of embryos were examined for stages near the time of onset of expression.
[b] A gene was considered to show significant expression if the levels of expression in the limb bud were higher than levels found in E11 heart, a tissue considered to be negative for MDFs.
[c] Earliest embryo stage in which significant levels of MDF expression were consistently detected in the forelimb.
[d] Hindlimb buds are too small to dissect cleanly at stage 15.
[e] Earliest embryo stage in which significant levels of MDF expression were consistently detected in the hindlimb.
[f] Significant levels of MyoD expression were consistently detected in both stage 23/24 forelimbs and hindlimbs.
[g] N.D., not determined.

The other three MDF PCR primers were also used to examine the stages at which transcripts from these genes could first be detected above background levels. Examples of results from these experiments are shown in Fig. 3, and the composite results from analyses for all four MDF transcripts are presented in Table 3. The MRF 4 primers produced a 283-bp PCR product that was found in stage 15 forelimbs and stage 16 hindlimbs after 29 PCR cycles, 4 cycles earlier than in E11 heart (Fig. 3A). MRF 4 transcripts, along with those of myf 5, were the earliest MDF mRNAs to be detected in the limb buds examined in this study. For technical reasons (i.e., minute limb bud size and limb bud proximity to somites), it was not possible to remove fore- and hindlimb buds prior to stages 15 and 16. MyoD transcripts were first found several stages later than myf 5 and MRF 4 transcripts in stage 23/24 fore- and hindlimbs. For example, in Fig. 3B, a stage 23 hindlimb sample exhibited a similar profile of MyoD RT–PCR amplification to that

observed in E11 heart. The 470-bp product was first seen in both samples after 25 cycles, thus implying that the stage 23 hindlimb contains background levels of MyoD transcript, despite the greater intensity of the stage 23 hindlimb MyoD PCR product than that of E11 heart. Indeed, the MyoD data from Table 3 suggests that stage 23 is the transitional period for MyoD activation since half the limb samples exhibited above background levels of MyoD transcripts in both the fore- and hindlimbs. In contrast, almost all of the stage 24 limb buds contained MyoD transcript levels that are greater than background (Table 3); for example, the stage 24 hindlimb sample depicted in Fig. 3B contained MyoD mRNA levels that were detected at least 3 cycles earlier than the background levels detected in E11 heart. A second set of MyoD primers confirmed that the temporal difference in MyoD transcript levels between limb stages was not due to gross differences in primer sensitivity, i.e., both sets of primers gave similar results with respect to the stages when the MyoD transcripts were first detected above background (data not shown).

With myogenin-specific PCR primers, the 205-bp myogenin PCR product was clearly visible in the E11 heart sample after 30 PCR cycles (Fig. 3C). The stage 24 forelimb exhibited a PCR cycling profile similar to that of E11 heart, thus indicating that it contained myogenin transcript levels at background levels. In contrast, the myogenin PCR product was seen in the stage 25 forelimb after only 25 cycles. This was significantly above background and suggests that myogenin gene expression has been activated in myogenic cells of stage 25 forelimbs. A third of the stage 24 forelimb bud samples examined showed significant levels of myogenin expression (Table 3). However, a majority of the stage 23 and 24 hindlimb buds exhibited myogenin mRNA levels above background. The slightly earlier detection of myogenin in hindlimb vs forelimb differed from the more equivalent timing of initial expression of the other three MDFs in the fore- and hindlimbs. Myogenin expression also appeared to rise above background slightly later than MyoD expression, and initiation of its expression more closely coincided with the onset of limb bud muscle differentiation.

Detection of myf 5, MRF 4, and MyoD Transcripts in Skeletal and Nonskeletal Muscle Tissues

In the analysis of limb bud MDF transcripts described in the previous section, we interpreted the detection of low levels of MDF transcripts in E11 heart as being due to ''leaky'' transcription. For example, MyoD PCR products were typically visible in heart samples after 25 cycles (Fig. 3B), whereas myf 5 mRNA was usually undetected in heart after 40 PCR cycles (Fig. 2A). Since the myf 5 primers can theoretically produce visible product from a single target molecule after 40 cycles (Table 2), these data suggest a lower level of leaky expression from the myf 5 gene relative to that of MRF 4, MyoD, and myogenin.

To assess MDF muscle specificity vs background MDF levels in other embryonic tissues, RT–PCR analysis of MyoD, myf 5, and MRF 4 mRNAs was carried out in samples from E11 heart, E11 liver, neural tubes from stage 9–15 embryos, lateral plate tissue (without ectoderm) from stage 9–12 embryos (Fig. 1A), and the distal and proximal regions of stage 25/26 limb buds (Fig. 1D, Table 4). Lateral plate tissue was included because it does not contain cells which fate map to the myogenic lineage, but it does give rise to the other mesodermal lineages of both the proximal and distal regions of the limb bud (Christ *et al.,* 1977). The distalmost 1/4 of the stage 25/26 limb bud was of interest, because previous clonal assays had indicated that this limb region is devoid of myogenic precursor cells (Rutz *et al.,* 1982). The identities of the PCR products amplified from all tissues were verified by restriction enzyme digests.

To make the distinction between positively regulated and leaky transcription rigorous, significant levels of MyoD, myf 5, or MRF 4 PCR products were defined as being greater than four times higher ($>$ 2 PCR cycles different) than those detected in parallel E11 heart samples. The tissue distribution of MyoD transcripts showed strong skeletal muscle specificity. An example of one experiment that contrasts MyoD and myf 5 expression in a variety of embryonic tissues is shown in Figs. 4 and 5. With the exception of the proximal stage 25/26 limb bud in which overt skeletal muscle differentiation is occurring, only background levels of MyoD mRNA were seen in all the other embryonic tissues tested (Fig. 4 and Table 4). In contrast, lateral plate, neural tube, and both distal and proximal portions of stage 25/26 limb buds contained significant levels of myf 5 transcripts when compared with heart (Fig. 5). E11 liver, like E11 heart, contained background levels of myf 5 transcripts.

Results with MRF 4 primers are summarized in Table 4 along with data from other experiments with MyoD and myf 5 primers in other embryonic tissues. The myf 5 and MRF 4 genes exhibited much less developmental and muscle-specific expression. Myf 5 products were not detectable after 40 PCR cycles in E11 heart and liver, whereas relatively abundant levels of myf 5 transcripts were detected in neural tube and lateral plate from earlier embryonic stages (Fig. 5). Similarly, MRF 4 transcripts were detected at significant levels in 4 of 7 neural tube samples as well as in 6 of 6 lateral plate samples. Significant myf 5 and MRF 4 expression were also detected in the proximal portion of stage 25/26 limb buds. This was expected since muscle differentiation in proximal limb regions is apparent at this stage. However, significant myf 5 expression was also detected in 9 of 11 distal 1/4 limb bud regions where myogenic cells are not thought to reside. MRF 4 expression in distal limb regions was more variable than that of myf 5, with only 7 of 11 stage 25/26 limb bud tips exhibiting significant MRF 4 transcript levels.

DISCUSSION

This study characterized the temporal appearance of MDF transcripts in developing chick fore- and hindlimbs using

TABLE 4
Detection of MyoD, myf 5, and MRF 4 Transcripts in Skeletal and Nonskeletal Muscle Tissue

Tissue	No. of samples examined	Samples at background levels[a]	Samples 3–4 cycles above heart levels	Samples ⩾5 cycles above heart levels
MyoD				
E 11 liver	6	6	0	0
Stage 9–15 neural tube	8	7	1	0
Stage 9–12 lateral plate	6	6	0	0
Stage 25/26 distal 1/4 forelimb buds	5	5	0	0
Stage 25/26 distal 1/4 hindlimb buds	6	6	0	0
Stage 25/26 proximal 3/4 forelimb buds	5	0	1	4
Stage 25/26 proximal 3/4 hindlimb buds	6	0	1	5
Myf 5				
E 11 liver	7	6	0	1
Stage 9–15 neural tube	8	1	0	7
Stage 9–12 lateral plate	6	0	0	6
Stage 25/26 distal 1/4 forelimb buds	5	0	0	5
Stage 25/26 distal 1/4 hindlimb buds	6	2	0	4
Stage 25/26 proximal 3/4 forelimb buds	5	0	0	5
Stage 25/26 proximal 3/4 hindlimb buds	6	0	0	6
MRF 4				
E 11 liver	5	5	0	0
Stage 9–15 neural tube	7	3	4	0
Stage 9–12 lateral plate	5	0	0	5
Stage 25/26 distal 1/4 forelimb buds	5	0	3	2
Stage 25/26 distal 1/4 hindlimb buds	6	4	1	1
Stage 25/26 proximal 3/4 forelimb buds	5	0	0	5
Stage 25/26 proximal 3/4 hindlimb buds	5	0	0	5

[a] Background levels of MDF transcripts in this analysis are defined as levels in which the specific PCR products are detected 2 or fewer cycles earlier than in E11 heart or later than E11 heart, a tissue considered negative for MDFs.

RT–PCR. Myf 5 and MRF 4 mRNAs were detected in the earliest forelimbs (stage 15) and hindlimbs (stage 16) that were feasible to examine. Detection of myf 5 and MRF 4 transcripts at stage 15 correlates with prior studies in which cells capable of muscle colony formation were detected in stage 15+ forelimbs (Seed and Hauschka, 1984). The initial appearance of MyoD and myogenin mRNA occurs later than myf 5 and MRF 4, but precedes the initial expression of muscle structural genes at stage 26 (Charles de la Brousse and Emerson, Jr., 1990). It is important to emphasize that the myf 5 and MRF 4 transcripts detected in limbs are not attributable to somite contamination. This conclusion is based on the fact that MyoD and myogenin mRNAs were not detected at levels above background in stage 15 and 16 limbs, and yet the potentially contaminating limb level somites already contain differentiated axial muscle in which these mRNAs are readily detected (Lin-Jones and Hauschka, unpublished observations).

Comparison of the appearance of MDF transcripts in forelimb vs hindlimb reveals no temporal difference as to when myf 5, MRF 4, and MyoD are first detected. Due to the rostral–caudal gradient of somite development, somites from which the forelimb muscles are derived form earlier than those of the hindlimb, and precursors to the forelimb muscles leave the somite a stage earlier than those of hindlimb muscles. Because myf 5 and MRF 4 transcripts were detected in the earliest limbs that can be dissected, it cannot be determined whether these mRNAs appear in the forelimb before the hindlimb. However, by stage 23, MyoD transcripts can be detected above background levels in both forelimb and hindlimb. The simultaneous expression of MyoD suggests that forelimb and hindlimb muscle development are equivalent by stage 23. However, the slight temporal difference between fore- and hindlimb with respect to the earliest detection of myogenin mRNA may indicate that terminal differentiation begins in hindlimb muscle about a stage prior to its initiation in the forelimb (Table 3).

The order of MDF expression in the chick limb detected by our studies differs from the sequence reported in mouse and avian somites and in mouse limbs as detected by *in situ* hybridization (Pownall and Emerson, 1992; see Lyons and Buckingham, 1992, for review of mouse data), and in mouse embryos as detected by RT–PCR (Hannon *et al.*, 1992). Our results show that myf 5 and MRF 4 are the first MDF transcripts in developing chick limbs and that these are present as early as stages 15 and 16 in the fore- and

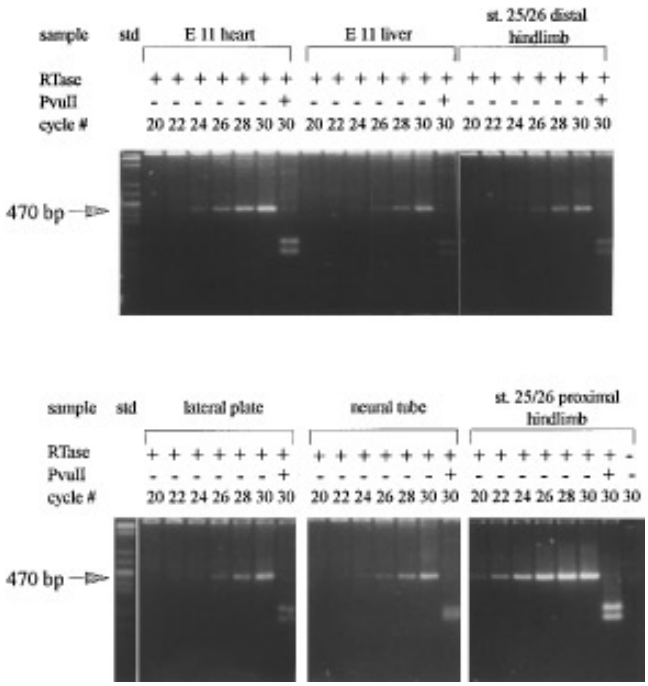

FIG. 4. Detection of MyoD transcripts by RT–PCR in embryonic muscle and nonmuscle tissues. The ''a'' set of MyoD PCR primers was used to amplify cDNA made from E11 heart and liver, lateral plate (without ectoderm) from stage 9–12 embryos, neural tube from stage 9–15 embryos, and the distalmost quarter and remaining proximal three-quarters of stage 25/26 hindlimbs. Background levels of MyoD transcripts, defined by E11 heart levels, were detected in E11 liver, lateral plate tissue, neural tube, and the distal portion of stage 25/26 hindlimb. These samples produced visible 470-bp MyoD PCR product after 22 or more cycles. Only the proximal portion of the stage 25/26 hindlimb exhibited the 470-bp MyoD product after 20 cycles. Verification of the PCR product as the targeted portion of MyoD cDNA was confirmed by restriction digest with *Pvu*II. No amplification was seen in the stage 25/26 proximal hindlimb sample after 30 cycles in the absence of reverse transcriptase (-RTase). All cDNA samples exhibited efficient amplification of the 409-bp cytoplasmic β-actin product and verified the integrity of the RNA samples (data not shown).

hindlimbs. Extensive *in situ* hybridization analysis of MDF transcripts in early chick limbs has yet to be reported, but the detection of MyoD mRNA in stage 24 limbs (Williams and Ordahl, 1994) is consistent with our RT–PCR data (Table 3). During avian somitogenesis, MyoD was the earliest MDF gene expressed as detected by *in situ* hybridization of stages 12 and 13 quail embryos (Pownall and Emerson, 1992). In that study, myf 5 and myogenin transcripts were detected in somites later than MyoD transcripts, whereas in our RT–PCR studies of chick limbs, MyoD and myogenin mRNAs were detected 8 stages or approximately 30 hr later than myf 5 and MRF 4 mRNA.

Comparison of chick limb RT–PCR data with mouse limb *in situ* hybridization and RT–PCR data indicates that the order of MDF transcript appearance is relatively similar between the two species. Myf 5 is the earliest MDF gene to be activated in both mouse somites and limbs (Ott *et al.*, 1991), while MyoD and myogenin are coexpressed later in the mouse limb (Sassoon *et al.*, 1989). One major discrepancy in the temporal expression of MDFs in mouse and chick limbs is the detection of MRF 4 transcripts. Chick MRF 4 mRNA, like myf 5, was found in the earliest limb stages, eight stages before MyoD and myogenin transcripts (Table 3). In mouse limbs, MRF 4 mRNA was not detected by *in situ* hybridization until 2–3 days after the other MDF mRNAs (Bober *et al.*, 1991; Hinterberger *et al.*, 1991). However, an RT–PCR analysis of mouse limbs showed MRF 4 transcripts present at the same early stages in which myf 5 transcripts were detected (Hannon *et al.*, 1992). This result is consistent with our findings in the chick limb as well as with the temporal pattern in mouse somites.

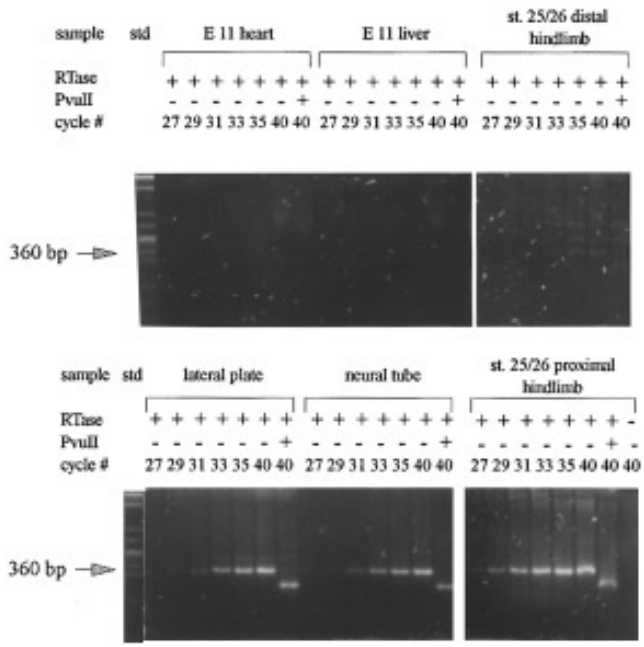

FIG. 5. Detection of myf 5 transcripts by RT–PCR in embryonic muscle and nonmuscle tissues. The ''a'' set of myf 5 PCR primers was used to amplify cDNA made from E11 heart and liver, lateral plate (without ectoderm) from stage 9–12 embryos, neural tube from stage 9–15 embryos, and the distalmost quarter and remaining proximal three-quarters of stage 25/26 hindlimbs. No background level of myf 5 was detected in E11 heart or liver even after 40 PCR cycles. The 360-bp myf 5 PCR product was visibly amplified with 40 or fewer cycles in samples from neural tube, lateral plate, and distal and proximal portions of stage 25/26 hindlimbs. Verification of the PCR product as the targeted portion of the myf 5 cDNA was confirmed by restriction digest with *Pvu*II. No amplification was seen with myf 5 primers in the absence of reverse transcriptase (-RTase) after 40 cycles. The cDNA samples used for amplification of the myf 5 PCR products shown in this figure were the same used for amplification of the MyoD PCR products (shown in Fig. 4) in the same experiment.

TABLE 5
Morphogenetic, RT–PCR, and Cell Culture Analyses of Chick Limb Muscle Development

H & H stage	Muscle morphogenesis	RT–PCR assays: myf 5	RT–PCR assays: MRF 4	RT–PCR assays: MyoD	RT–PCR assays: Myogenin	Cell culture assays
14	Cells from lateral edge of somite begin migration into lateral plate mesoderm at the wing level[a]	N.D.[b]	N.D.	N.D.	N.D.	
15	Somitic cells begin migration into the leg bud[c]	+ (*f*)[d]	+ (*f*)	—	—	Heterotopic wing bud transplants indicate presence of early type MCF[e] cells[f]
16	Myogenic precursor cells indistinguishable from surrounding limb mesenchyme	+ (*f* & *h*)[g]	+ (*f* & *h*)	—	—	Premyogenic cells are detected in the wing bud by explant cultures[h]
17		+ (*f* & *h*)	+ (*f* & *h*)	—	—	Late type MCF cells present in wing,[f] premyogenic cells detected in leg bud by explant cultures[i]
21 and 22	Myogenic regions of limb become visible[j]	+ (*f* & *h*)	+ (*f* & *h*)	—	—	Early type MCF cells detected in leg buds[k]
23	Dorsal and ventral limb premuscle regions become histologically visible[j]	+ (*f* & *h*)	+ (*f* & *h*)	± (*f* & *h*)	± (*h*)[m]	MCF cells distributed in proximal–distal gradients within dorsal and ventral limb premuscle regions[n]
24		+ (*f* & *h*)	+ (*f* & *h*)	+ (*f* & *h*)	± (*f* & *h*)	Detection of fast, slow, and fast/slow MCF types[o]
25	Premuscle mass regions begin splitting into anatomically discrete muscles[p]	+ (*f* & *h*)	+ (*f* & *h*)	+ (*f* & *h*)	+ (*f* & *h*)	Late type MCF cells first detected in leg bud[k]
26 and later	Myotube fusion commences[j]	+ (*f* & *h*)	+ (*f* & *h*)	+ (*f* & *h*)	+ (*f* & *h*)	Late MCF cells increase while early MCF cells decrease with limb age[k]

[a] Christ *et al.* (1977).
[b] N.D., not determined.
[c] Jacob *et al.* (1979).
[d] Forelimb bud.
[e] MCF cells, muscle colony-forming cells (see Seed and Hauschka 1984).
[f] Seed and Hauschka (1984).
[g] Fore- and hindlimb buds.
[h] Rutz and Hauschka (1983).
[i] Dientsman *et al.* (1974).
[j] Hilfer *et al.* (1973).
[k] White *et al.* (1975).
[l] Pautou (1977), Rutz *et al.* (1982).
[m] Hindlimb bud.
[n] Rutz *et al.* (1982).
[o] Miller and Stockdale (1986).
[p] Romer (1927), Shellswell and Wolpert (1977), Pautou *et al.* (1982), Schroeter and Tosney (1991).

Myf 5 gene expression in mouse limb development has also been studied in chimaeric embryos containing cells with a lacZ cDNA inserted into the endogenous myf 5 locus (Tajbakhsh and Buckingham, 1994). The earliest β-galactosidase staining occurred in E10 limbs, and no β-galactosidase activity was found in the lateral plate mesoderm or in the earliest limbs, even though myogenic precursors are known to be present within the E9.25 limbs (Sassoon *et al.,* 1989). These data are consistent with the hypothesis that myf 5 is not expressed in muscle precursors of the earliest mouse limbs. However, this interpretation also depends on the assumptions that insertion of the lacZ cDNA did not disturb any temporal controls of myf 5 gene expression and that all of the early limbs contained high and uniform levels of chimaerism (myf 5-lacZ and wild-type cells).

The MyoD PCR primers used in our study and the *in situ* hybridization probes used by other investigators exhibit roughly equivalent levels of sensitivity since our assays first detected MyoD mRNA at the same stage as observed by *in situ* hybridization. In contrast, our myf 5 and MRF 4 PCR primers appear to be much more sensitive than the probes used for the *in situ* hybridization studies, i.e., myf 5 and MRF 4 mRNAs were detected in tissues that were negative for myf 5 and MRF 4 mRNA as assessed by *in situ* hybridization (Ott *et al.,* 1991; Bober *et al.,* 1991; Hinterberger *et al.,* 1991).

Detection of mRNAs by *in situ* hybridization is highly dependent on the signal to noise ratio. In the case of radioactive *in situ* hybridization probes, background noise could be due to the combination of specific probe hybridization to genomic DNA sequences, low levels of legitimate MDF transcripts from leaky gene transcription, nonspecific hybridization, and random autoradiographic grain exposure. In contrast, when intron-spanning PCR primers are used and PCR product identity is verified by diagnostic restriction enzyme digest, the only obvious contributor to background noise is the presence of mRNAs due to leaky gene expression. ''Leaky'' transcription in tissues in which the particular gene is not thought to function has been found for a number of genes (Chelly *et al.,* 1988; Montarras *et al.,* 1989). For example, β-globin mRNA was detected in fibroblast, hepatoma, and lymphoblast cell lines by RT–PCR (Chelly *et al.,* 1989).

It was critical for our study to use a non-skeletal muscle tissue such as E11 heart as the negative control for leaky gene transcription in exactly parallel RT–PCR assays to determine the earliest PCR cycle number at which background transcripts were observed. For MyoD, extrapolation of the data from Table 2 and Fig. 3B suggests that approximately 30,000 transcripts of MyoD (1 transcript for every 3 cells) are found in 1 μg of total RNA from E11 heart. Similar calculations for background levels of MRF 4 (Fig. 3A and data not shown) suggest that there are approximately 1000 transcripts in 1 μg of E11 heart RNA (1 transcript for every 100 cells). In contrast, myf 5 PCR products can be detected only occasionally in E11 heart after 40 cycles, even though a single myf 5 target sequence can be detected in PCR sensitivity assays after 40 cycles (Table 2) (see below for further interpretation of low level MDF transcripts).

Positively regulated transcription in our assay was defined as PCR products detected two or more cycles prior to their detection in parallel samples from the nonskeletal muscle control tissue (E11 heart). Therefore, because of the differences in background transcription levels between myf 5, MRF 4, and MyoD, the number of target sequences required for significant transcript levels in this assay would differ for each of the MDFs. For example, a significant myf 5 signal would require the presence of only a few target sequences, while the detection of a significant MyoD signal would require a much greater number of target sequences. These differences in background MDF mRNA levels may be responsible for the greater sensitivity of myf 5 and MRF 4 RT-PCR assays.

We examined MyoD, myf 5, and MRF 4 transcript levels in other chick embryonic tissues by RT–PCR and found unanticipated patterns of developmental and tissue expression. Significant levels of MyoD transcripts were found only in tissues containing skeletal muscle or skeletal muscle precursors (Fig. 4 and Table 4), whereas significant myf 5 and MRF 4 levels were detected in these tissues as well as in tissues not known to contain skeletal muscle precursors. For example, significant levels of myf 5 and MRF 4 mRNA were found in early neural tubes, an observation that corroborated those from myf 5-lacZ insertion studies that showed myf 5 expression in the mouse embryo neural tube (Tajbakhsh *et al.,* 1994).

Detection of myf 5 and MRF 4 transcripts in lateral plate is of interest, because this tissue contains precursors to bone, cartilage, connective tissue, tendons, and smooth muscle cells that are found within the developing limb (Christ *et al.,* 1977, 1979). Transplants of quail lateral plate mesoderm into chick hosts have yielded conflicting results as to whether lateral plate mesoderm can form muscle (Christ *et al.,* 1977, 1979; McLachlan and Hornbruch, 1979; Mauger *et al.,* 1980; Wachtler *et al.,* 1982). While most studies support an exclusively somitic origin of skeletal muscle cells, the role of myf 5 and MRF 4 in determining the myogenic potential of transplanted lateral plate mesoderm is unclear.

Detection of myf 5 and MRF 4 mRNA in the distal limb does not correlate with the presence of muscle precursors, since no myogenic cells were detected within this region of stage 25 limbs by cell culture assays (Hauschka and Rutz, 1982; Rutz *et al.,* 1982). The apparent absence of skeletal muscle precursors in lateral plate mesoderm and distal limb regions suggests that myf 5 and MRF 4 expression within these cells does not cause an irreversible commitment to myogenesis. If expression of myf 5 and MRF 4 in these tissues does, indeed, provide the potential for initiating early phases of myogenesis, it is possible that further activation can occur only when the proper environmental conditions are present.

The detection of myf 5 and MRF 4 transcripts in the nonmyogenic distal limb region as well as in the lateral

plate has important interpretive consequences for our data. Namely, we cannot distinguish whether the myf 5 and MRF 4 transcripts detected in early limbs are due solely to nonmyogenic limb mesenchyme cells derived from the lateral plate, or to both nonmyogenic and myogenic cell populations. However, even if higher relative MDF transcript levels could be localized to specific cells with myogenic potential, as with *in situ* hybridization, it would still remain to be determined whether the transcripts corresponded to functional levels of MDF proteins, i.e., are the transcripts translated, and are the MDF proteins in a functionally activated state with respect to regulating muscle gene expression? While it is tempting to use this PCR data to estimate absolute concentrations of MDF transcripts per limb cell, we believe that too many untested assumptions would be required to derive such values. However, biologically significant mRNA levels for certain liver enzymes have been estimated to be as low as two molecules per cell (Galau *et al.*, 1977).

The enigma of early and seemingly inappropriate MDF gene expression is further illustrated by the detection of myf 5 mRNA in stage 3 chick embryos, while no myf 5 mRNA was present at stage 1 (Lin-Jones and Hauschka, manuscript in preparation). This observation suggests that the myf 5 gene is activated during early gastrulation. However, its transcription must be subsequently repressed in nonmyogenic tissues, since myf 5 transcripts were barely detectable in E11 heart and liver. An analogous pattern of widely distributed, early MDF expression, followed by progressively restricted expression, is seen during *Xenopus* development (Rupp and Weintraub, 1991). MyoD transcripts are distributed throughout the embryo at the mid-blastula transition; then as development proceeds, MyoD expression becomes restricted to cells of the muscle lineage.

Before the discovery of MDFs heterotopic limb transplants, limb explant cultures, and limb clonal cell culture assays were used to determine the presence of cells committed to the muscle lineage (see Table 5 footnotes for references). These earlier studies are summarized and compared to our current RT–PCR results in Table 5. A stage after cells from the lateral edge of the somite begin their migration to the limb bud, myogenic precursors can be detected in heterotopic transplants of stage 15 forelimbs (Seed and Hauschka, 1984). This is a time when myf 5 and MRF 4 mRNAs are also detected in forelimbs. Between stages 15 and 17 at least two myogenic precursor types appear (Seed and Hauschka, 1984), and some of these cells may already differ in their bias toward fast and slow muscle fiber formation (Van Swearingen and Lance-Jones, 1995). It is of interest that limb explants and clonal assays of such early limb cells are able to detect premyogenic limb cells well before MyoD and most structural muscle genes are expressed. This suggests that at least some of the cells containing myf 5 and MRF 4 transcripts are committed to the myogenic lineage.

During the next 1.5 days, myf 5 and MRF 4 expression continues while MyoD and myogenin expression levels remain at background. Throughout this period the limb myogenic population increases, and the cells undergo at least one phenotypic change that can be detected in cell culture. During the stage 16–20 period myogenic cells are readily observable via explant culture assays, but not via clonal assays (Hauschka and Rutz, 1982). This behavior changes at stage 21 when muscle colony-forming cells first appear (Bonner and Hauschka, 1974). Two stages later, MyoD and myogenin mRNAs appear, followed shortly by transcripts associated with terminal muscle differentiation as exemplified by the appearance of troponin T mRNA in stage 26 limbs (Charles de la Brousse and Emerson, Jr., 1990). The later appearance of myogenin transcripts is consistent with data from myogenin null mutations in mice (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993) as well as with the pattern of myogenin expression in muscle culture (Edmondson and Olson, 1989; Wright *et al.*, 1989) and suggests the importance of myogenin for muscle terminal differentiation. MyoD may play a similar role to myogenin in terminal differentiation, but it may also play a role in establishing the myoblast subtypes that appear during subsequent embryogenesis, e.g., the late muscle colony-forming cells found in stage 25 hindlimbs (Rutz *et al.*, 1982) or the colony-forming cells that express different myosin heavy chain isoforms (Miller and Stockdale, 1986).

This study has demonstrated that two muscle determination factor transcripts, myf 5 and MRF 4, are detected in the earliest stages of limb development and in a variety of myogenic as well as nonmyogenic embryonic tissues by RT–PCR. Myogenin and MyoD transcripts are found only during later limb stages and appear to be associated with steps in myogenesis that slightly precede and/or coincide with terminal differentiation. RT–PCR studies to examine the developmental sequence of MDF gene expression during somitogenesis are currently in progress and should determine whether similar patterns of early and late MDF gene expression also occur during myogenesis in the somite.

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